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ORIGINAL ARTICLE

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Analysis of *N*-alkylated amino acids in human hemoglobin: evidence for elevated *N*-methylvaline levels in smokers

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Abstract To investigate the contribution of cigarette smoking to the levels of *N*-methylvaline and *N*-hydroxyethylvaline in hemoglobin we analyzed samples from 32 smokers and 37 nonsmokers. The average background levels of the nonsmoking individuals were determined to be 1175 ± 176 pmol *N*-methylvaline/g globin, ranging from 722 to 1516 pmol/g globin, and 46 ± 12 pmol *N*-hydroxyethylvaline/g globin, ranging from 19 to 64 pmol/g globin. A significant correlation ($P < 0.001$) was found between both amino acids and the amount of cigarettes smoked per day, with an increase of 42 pmol *N*-methylvaline/g globin/cigarette per day and 11 pmol *N*-hydroxyethylvaline/g globin/cigarette per day. No influence of age, sex, and occupational exposure was observed. Furthermore, the levels of *N*-hydroxyethylvaline and *N*-methylvaline correlated for smokers but not for nonsmokers, indicating cigarette smoking as a common source for both adducts. To our knowledge, this is the first time *N*-methylvaline levels are reported to differ significantly between smokers and nonsmokers in the general population. Especially the analysis of *N*-methylvaline following low-level exposures to methylating agents should therefore take into consideration the influence of individual smoking habits. Additionally, the results of our study confirm the reliability of *N*-hydroxyethylvaline as an indicator of individual cigarette consumption. We successfully applied a new calibration technique to the analysis of *N*-hydroxyethylvaline, introducing a commercially available and well-defined dipeptide standard. The observed levels of *N*-hydroxyethylvaline in the samples are in line with those reported in the literature and verify the applicability of our calibration method.

Key words Biomonitoring · Hemoglobin adducts · *N*-alkyl Edman method · Tobacco smoking · Gas chromatography/mass spectrometry

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Introduction

The formation of blood protein adducts has been intensively investigated in the course of the development of protein biomarkers. In many cases, protein adducts have been shown to reflect the internal strain caused by hazardous chemicals. Though blood protein adducts do not necessarily exert a biological effect by themselves, they correlate with the internal availability of a reactive and thereby toxic metabolite of a chemical. As a consequence, they can be regarded as an indirect measure for toxic effects and provide a parameter for biochemical effect monitoring (Neumann 1988).

Some applications are routinely used for the monitoring of occupational exposures, e.g. simple epoxides (Kautiainen and Törnqvist 1991), aromatic amines (Sabbioni 1992), and polycyclic aromatic hydrocarbons (Day et al. 1991). Background adduct levels are usually observed for chemicals with endogenous or environmental sources. Additionally, individual smoking habits play an important role in adduct formation due to the presence of various reactive species in the tobacco smoke. Though the resulting background adduct levels are of general interest for dose-response calculations in risk assessment, they are likely to hinder the analysis of low-level occupational exposures.

Usually, hemoglobin is chosen for adduct analysis for both fundamental and practical reasons (e.g., Ehrenberg and Osterman-Golkar 1980; Garner 1985; Skipper and Tannenbaum 1990; Skipper et al. 1994). Different methods for the analysis of modifications of

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the most frequently adducted amino acids have been established. Especially in the case of simple epoxides the *N*-alkyl Edman method based on the cleavage and subsequent derivatization of the N-terminal valine has been further developed in recent years. This method combines a relatively simple analytical procedure and a good reproducibility with sufficient sensitivity for multiple applications, e.g. the analysis of adducts of methylating agents (Törnqvist et al. 1988a), ethylene and propylene oxide (Törnqvist et al. 1988b), styrene oxide (Christakopoulos et al. 1993) and butadiene (Osterman-Golkar et al. 1991).

Background alkylations are reported especially for *N*-methylvaline, *N*-hydroxyethylvaline, and some short-chained aldehydes (Törnqvist 1991). Furthermore, *N*-hydroxyethylvaline was the first adduct in this series to show a significant increase in the adduct level dependent on tobacco smoking caused by ethene and ethylene oxide in the tobacco smoke (Bailey et al. 1988). Cigarette smoking could not be related to the formation of adducts originating from methylating components of tobacco smoke such as formaldehyde, methyl chloride, or *N*, *N*-dimethylnitrosamine. This result is due to the high background of *N*-methylvaline probably generated by the action of *S*-adenosylmethionine (Törnqvist et al. 1988a). In contrast, a recent study of monozygotic twin pairs differing in their smoking habits reported by Törnqvist et al. (1992a) revealed an influence of both hereditary and tobacco-related factors on *N*-methylvaline levels.

In order to investigate the applicability of the Edman method in the differentiation of smokers and nonsmokers in the general population, we chose a group of 32 smokers and 37 nonsmokers for the analysis of *N*-methylvaline. Additionally, *N*-hydroxyethylvaline was analyzed to confirm the reliability of the obtained data. A further aspect of our study was the introduction of a new calibration technique for the routine analysis of *N*-hydroxyethylvaline and to prove its suitability for future applications of the *N*-alkyl Edman method.

Materials and methods

All chemicals were of the best available analytical grade and were obtained from Merck (Darmstadt, Germany) and Sigma (Deisenhofen, Germany) except for formamide (ultrapure, USB, Bad Homburg, Germany) and pentafluorophenyl isothiocyanate (Fluka, Buchs, Switzerland). DL-*N*-methylvaline and DL-pipecolic acid were purchased from Sigma (Deisenhofen, Germany). The *N*-hydroxyethylvaline-leucine-anilide (purity > 98%, determined by thin-layer chromatography) was synthesized and specified by Bachem AG (Bubendorf, Switzerland).

Study

The blood samples were obtained in an earlier study (Letzel et al. 1993) from 42 employees of a refinery plant working in the production of butadiene. The workers were protected by charcoal gas masks. The control group consisted of 27 nonexposed persons from the same plant. A detailed collection of anamnestic data was carried out especially with regard to the individual smoking habits.

Personal ambient air monitoring was performed, with results ranging from < 1 mg butadiene/m^3 to 23 mg butadiene/m^3 with a maximum value of 72 mg butadiene/m^3. No butadiene was detectable in postshift blood and exhaled air. Additionally, the induction of micronuclei, sister chromatid exchanges, DNA single strand breaks, and DNA repair were investigated in the same study, but no significant differences were found between the exposed workers and the control persons. A descriptive survey of the study population is given in Table 1.

Table 1 Study population

	Total	Nonsmokers	Smokers
No. of persons	69	37	32
Male/female	42/27	25/12	17/15
Average age	42	43	42
Range	24–61	28–61	24–55
Average cig./day			12.3
Range			2–30
Exposed	42	15	27
Male/female	25/17	10/5	15/12
Controls	27	22	5
Male/female	17/10	15/7	2/3

Blood sampling and isolation of globin

Whole blood (5 ml) was collected from each person by venipuncture using EDTA-containing syringes (Sarstedt, Nümbrecht, Germany). The blood was centrifuged for 10 min at 800 × *g* and the supernatant plasma was discarded. The erythrocytes were washed three times with 5 ml of 0.9% NaCl solution. Hemolysis was achieved by adding 3 ml of distilled water. The fragments of the cell membranes were removed by centrifugation at 5°C for 30 min at 30 000 × *g*. To prepare about 150 mg of globin from a sample, 2 ml of each hemolysate were added to 12 ml of a 50 mM hydrochloric acid solution in 2-propanol. The samples were stored for at least 1 h at 4°C and subsequently centrifuged for 10 min at 3500 × *g*. Ethyl acetate (8 ml) was added to the supernatant and the solution was stored at 4°C overnight. The precipitated globin was spun down for 10 min at 3500 × *g* and washed three times with 5 ml of ethyl acetate. Finally, the globin was washed with 5 ml of *n*-hexane and dried in a vacuum desiccator.

Edman derivatization procedure

The *N*-alkylated amino acids were analyzed by gas chromatography/mass spectrometry (GC/MS) following a slightly modified Edman procedure as described by Törnqvist et al. (1986a). In detail, 100 mg of each globin sample were dissolved in a solution of 3 ml of formamide, 30 µl of 1 N NaOH and 50 µl of internal standard (150 µg/l DL-pipecolic acid in formamide) and sonified for 30 min. 10 µl of pentafluorophenyl isothiocyanate were added and the samples were rotated overnight at room temperature, followed by an incubation for 90 min at 45°C. The solutions were extracted twice with 3 ml of diethylether and the combined organic layers were evaporated to dryness under a gentle stream of nitrogen. The residues were dissolved in 2 ml of toluene. The samples were washed with 2 ml of distilled water and subsequently with 2 ml of a freshly prepared 0.1 M sodium carbonate solution. The toluene layer was evaporated under a stream of nitrogen and the residue was redissolved in 30 µl of toluene. 1 µl of each sample was then analyzed by GC/MS.

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Calibration standards

Calibration curves for *N*-methylvaline were prepared in the range from 1 to 10 µg/l. Briefly, a stock solution of *N*-methylvaline (1000 mg/l in methanol) was diluted to 10 µg/l, 5 µg/l and 1 µg/l in formamide. 100 mg of a pool globin from blood samples of 70 control persons from an earlier study were dissolved in 3 ml of each standard solution and treated as described above for the study samples.

In the case of *N*-hydroxyethylvaline, 34.9 mg of the dipeptide standard, *N*-hydroxyethylvaline-leucine-anilide, were dissolved in 20 ml of methanol, yielding a concentration of *N*-hydroxyethylvaline of 5 mM. This solution was diluted to 500, 250, and 50 nM in methanol. 50 µl of these standard solutions were used to spike 100 mg of pool globin samples in 3 ml of formamide, 30 µl NaOH, and 50 µl of internal standard, resulting in concentrations of 250, 125, and 25 pmol *N*-hydroxyethylvaline/g globin.

Analytical determination

One microliter of each toluene solution was injected splitless into the gas chromatograph. The pentafluorophenyl thiohydantoins of *N*-methylvaline, *N*-hydroxyethylvaline, and pipecolic acid were identified by their retention times and quantified by two characteristic masses: 338/310 amu (*N*-methylvaline), 350/308 amu (*N*-hydroxyethylvaline), and 336/308 amu (pipecolic acid). The amounts of *N*-methylvaline and *N*-hydroxyethylvaline in the globin samples were calculated from the calibration curves.

GC/MS conditions

Gas chromatograph: Hewlett Packard 5890 series II; capillary column: Chrompack fused silica DB-5, 60 m × 0.22 mm, 0.33 µm film thickness; carrier gas: helium 5.0; column pressure: 15 PSI; temperatures: injector 280°C, column: 120°C, increase of 10°C/min to 280°C, isothermal for 19 min, transfer line 300°C, ion source 200°C, quadrupole. 100°C; ion source pressure: 2×10^{-6} Torr; ionization mode: electron impact (EI), electron energy: 70 eV; detection mode: selected ion monitoring (SIM), dwell time: 100 ms/ion trace, electron multiplier: 2800 V.

Statistical analyses

The correlations between the amount of cigarettes smoked per day and the adduct levels were calculated from linear regression analyses, as well as the dependency on age. To estimate the contribution of sex, exposure, and the smoking habits to the adduct levels, these parameters were analyzed within the framework of a multivariance analysis. Furthermore, a "resolution" of each method was calculated which indicated the minimum number of cigarettes smoked daily that could be differentiated from the background level. This procedure is similar to the mathematical determination of the detection limit of a method by repeated background level analysis (DIN 1991).

Results and discussion

We determined *N*-hydroxyethylvaline to confirm the individual declarations about smoking habits. This parameter is of high diagnostic validity since nonsmoker values are low and smoking exerts a well-defined influence on the adduct levels. An *N*-alkyl Edman method was established in our laboratory, introducing a new dipeptide standard for calibration. We modified the usual sample treatment by starting with more globin, making changes in the volumes of the extraction solutions, and reducing the number of washing steps compared to the workup by Törnqvist et al. (1986a). The modifications allow the analysis of background levels of *N*-hydroxyethylvaline with electron ionization instead of negative chemical ionization (NCI). NCI usually increases the sensitivity of the Edman method by a factor of 10 in this case but is more susceptible to analytical interferences, impurities, and instrumental problems.

For the nonsmoking individuals, we determined an average background level of 46 pmol/g globin, ranging form 19 to 64 pmol/g globin. This result is in line with levels usually reported in the literature, as shown in Table 2. The theoretical resolution of the method was calculated from the background values to be 1.9 cigarettes. This value indicates the number of cigarettes smoked per day that cause a statistically significant increase in the *N*-hydroxyethylvaline level, at least for group-based calculations. An increase of 11 pmol/g globin/cigarette per day was obtained from the regression curve between *N*-hydroxyethylvaline and the cigarettes smoked per day (Fig. 1). This value corresponds well with those reported from other studies. Of 32 smokers, 27 (84%) revealed *N*-hydroxyethylvaline levels above the highest nonsmoker value and 30 (94%) exceeded the 95th percentile of the nonsmokers.

The results of our study confirm both the validity of *N*-hydroxyethylvaline as a parameter for individual smoking habits and the correspondence of our values with those generally reported in the literature. This aspect is of great interest since we introduced a dipeptide standard for the calibration of the adduct level. Usually globins with a defined content of hydroxyethylated valine serve as calibration standards for the *N*-alkyl Edman method (Mowrer et al. 1986). These modified proteins are difficult to synthesize and to specify. Since the dipeptide standard we used is commercially available and of high certified purity, further work will be carried out in our laboratory to extend the applicability of this method to other adducts, e.g., *N*-methylvaline.

Table 3 displays the results for *N*-methylvaline and selected reference values quoted in the literature. An average background level for nonsmokers of 1175 pmol/g globin, ranging from 722 to 1516 pmol/g globin, was found. This value exceeds the generally reported adduct levels as a result of the calibration by "free" *N*-methylvaline applied in our study. Since the pK_a values of protein-bound *N*-alkylated amino acids are about two units lower than those of nonbound amino acids, the derivatization yield of the Edman procedure at neutral pH is higher for the

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Table 2 Levels of *N*-hydroxyethylvaline in globin samples from smokers and nonsmokers determined in different studies ($\bar{x} \pm SD$ mean ± standard deviation, *m* median, *95%* 95th percentile; *n* number of samples, *n.d.* not determined.)

References	Nonsmokers (pmol/g globin) $\bar{x} \pm$ SD m; 95% range (*n*)	Smokers (pmol/g globin) $\bar{x} \pm$ SD m; 95% range (*n*)	Increase (pmol/g globin/ cig. per day)
Törnqvist et al. (1986b)	58 ± 25 –;– 27–106(14)	389 ± 138 –;– 217–690(11)	n.d.
Bailey et al. (1988)	50 ± 24 56;101 22–106(24)	200 ± 113 167;458 38–510(26)	7.1
Sarto et al. (1991)	24 ± 2 24;– 21–26(4)	148 ± 114 88;– 76–280(3)	10.3
Törnqvist et al. (1992a)	16 ± 7 14;– 9–29(10)	147 ± 82 128;– 50–355(10)	9.4
This study	46 ± 12 47;63 19–64(37)	171 ± 93 144;318 31–327(32)	11

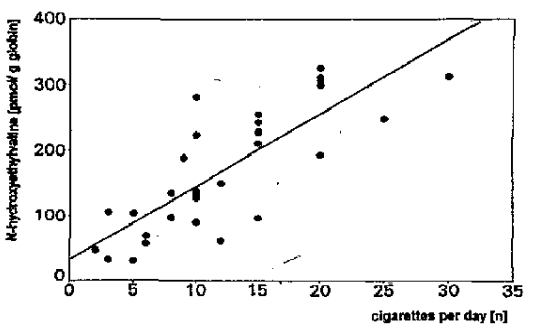

Fig. 1 Levels of *N*-hydroxyethylvaline from the smoker samples related to the daily number of cigarettes smoked [regression line: $y = 11$ pmol/(g globin × cig. per day) + 33 pmol/g globin, $P < 0.001$, $r = 0.804$]

protein-bound species (Törnqvist et al. 1986a). In the case of *N*-hydroxyethylvaline, a factor of about 8 was reported (Törnqvist et al. 1992b), and this should also be more or less the case for *N*-methylvaline. Nevertheless, the systematic overestimation of the adduct level does not influence the applicability of the method for the measurement of relative differences between two groups, e.g., smokers and nonsmokers.

We determined the average level of *N*-methylvaline for the smokers to be 1546 pmol/g globin, which is significantly higher ($P < 0.001$) than the result for the nonsmokers. This was unexpected to some extent, since literature reports and earlier studies by our laboratory (Törnqvist 1991; Bader and Angerer 1994) revealed no difference between smokers and nonsmokers. The first indication of a dependency of *N*-methylvaline levels on

Table 3 Levels of *N*-methylvaline in globin samples from smokers and nonsmokers determined in different studies ($\bar{x} \pm SD$ mean ± standard deviation, *m* median, *95%* 95th percentile; *n* number of samples, *n.d.* not determined.)

References	Nonsmokers (pmol/g globin) $\bar{x} \pm$ SD m; 95% range (*n*)	Smokers (pmol/g globin) $\bar{x} \pm$ SD m; 95% range (*n*)	Increase (pmol/g globin/ cig. per day)
Törnqvist et al. (1988a)	498 ± 102 550;– 360–630(13)	539 ± 90 560;– 370–650(7)	n.d.
Törnqvist et al. (1992a)	219 ± 33 229;– 166–284(10)	271 ± 33 272;– 217–372(10)	3.52
This study[a]	1175 ± 176 1162;1460 722–1516(37)	1546 ± 432 1425;2656 938–2702(32)	42

[a] Calibrated with "free" *N*-methylvaline

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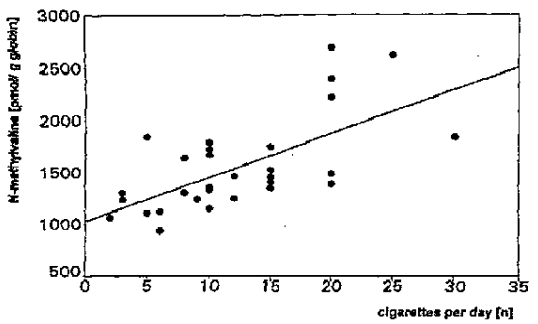

Fig. 2 Levels of *N*-methylvaline from the smoker samples related to the daily number of cigarettes smoked [regression line: $y = 42$ pmol/(g globin × cig. per day) + 1025 pmol/g globin, $P < 0.001$, $r = 0.649$]

smoking habits was presented by Törnqvist et al. (1992a) in a study with monozygotic twin pairs differing in their smoking habits. Figure 2 displays the *N*-methylvaline levels in relation to the daily number of cigarettes smoked. A gradient of 42 pmol/g globin/cigarette per day was calculated. Considering the systematic overestimation by a factor of about 8, this increase is in rough accordance with the results from the twin study (3.52 pmol/g globin/cigarette per day). The theoretical resolution of the method is 8.7 cigarettes per day. The *N*-methylvaline levels of only 12 smokers (38%) were above the highest nonsmoker value and 14 smokers (44%) were above the 95th percentile of the nonsmokers.

Thus, the determination of *N*-methylvaline levels is not suitable for the analysis of individual smoking habits and our study confirms earlier statements on this subject (Törnqvist et al. 1988a). Nevertheless, the results indicate that the influence of tobacco smoking on the *N*-methylvaline level is not negligible. The analysis of low-level exposures to methylating agents by the Edman method as occasionally reported (Lewalter et al. 1993) should consequently include a careful and precise analysis of the individual smoker status unless heavy smoking is likely to give the misleading impression of an occupational exposure.

In this context the dependency of the adduct levels on smoker status, sex, and the external exposure to butadiene was analyzed by a multivariance calculation (Table 4). A linear regression analysis was performed for the age dependency. Except for smoking, no significant correlation with the *N*-methylvaline and *N*-hydroxyethylvaline levels was found for any parameter. This result is of particular interest with respect to a possible influence of occupational butadiene exposure on the adduct levels. But as was confirmed by the biological monitoring of the study population and as was expected from the wearing of the breathing masks, no observable effect followed the exposure. Thus, the results of both the statistical analysis and the biological

Table 4 Multivariance analysis of the influence of different parameters on the adduct levels (+ and − signs indicate significance and absence of significance, respectively, at the $P < 0.05$ level)

Parameter	*N*-hydroxyethylvaline	*N*-methylvaline
Smoking	+	+
Sex	−	−
Age[a]	−	−
Butadiene exposure	−	−

[a] Calculated from a linear regression analysis

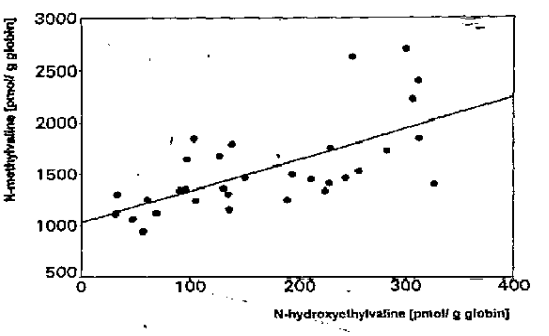

Fig. 3 Correlation between *N*-methylvaline (MEV) and *N*-hydroxyethylvaline (HEV) from the smoker samples [regression line: $y = 3$ (pmol MEV/g globin)/(pmol HEV/g globin) + 1029 pmol MEV/g globin, $P < 0.001$, $r = 0.647$]

monitoring qualify the chosen samples for a smoker/nonsmoker study.

Several factors may contribute to the unambiguous result of this study. Firstly, the number of persons chosen for this study ($n = 69$) improves the resolving power of the statistical analyses. A large number of persons is necessary since the effect of smoking on the level of *N*-methylvaline is quite small. Secondly, we found a good correlation between the results of the study and the individual declarations on smoking habits. Like most anamnestic parameters, this information is not wholly reliable, but it seems to have been quite accurate in this study, as it was confirmed by the results for *N*-hydroxyethylvaline. A third aspect worthy of mentioning is the small interindividual variation of *N*-methylvaline (about 15%) for the nonsmoking individuals and the absence of larger numbers of outliers in this study, which makes possible the detection of small changes caused by cigarette smoking. A variation of about 25%–35% is usually found (Törnqvist et al. 1988a).

Figure 3 displays the relationship between *N*-methylvaline and *N*-hydroxyethylvaline in the samples from the smoking individuals. The correlation of 3 pmol *N*-methylvaline/g globin per 1 pmol *N*-hydroxyethylvaline/g globin is significant and indicates cigarette smoking as a common source for the formation

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of these adducts. In contrast, no significant correlation was observed when the nonsmokers were analyzed separately (data not shown), indicating that the background levels of *N*-methylvaline and *N*-hydroxyethylvaline are independent of each other. This result contradicts findings in the twin study of Törnqvist et al. (1992a), who reported a correlation between the two parameters for nonsmokers and suggested a common link in the metabolic detoxification processes. The data from our study provide no evidence for this hypothesis. Reasons other than metabolic ones should also be taken into account in explaining the contradictory results, e.g., passive smoking or dietary sources.

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